Isolation of Nucleic Acids

This application is a continuation-in-part of USSN
09/586009, filed 2 June 2000, which derives from
5   PCT/GB98/03602, filed 4 December 1998, which claims
priority from UK patent application numbers 9725839.6,
filed 6 December 1997, and 9815541.9, filed 17 July 1998.
The entire disclosure of the '009 application is incorporated
by reference herein.
The present invention relates to a method for extracting
10   nucleic acids and other biomolecules from biological
materials, particularly blood and other liquid samples.

There is a very large demand for DNA analysis for a range
of purposes and this has lead to the requirement for
15   quick, safe, high throughput methods for the isolation
and purification of DNA and other nucleic acids.

Samples for use for DNA identification or analysis can be
taken from a wide range of sources such as biological
20   material such as animal and plant cells, faeces, tissue
etc. also samples can be taken from soil, foodstuffs,
water etc.

Existing methods for the extraction of DNA include the
25   use of phenol/chloroform, salting out, the use of
chaotropic salts and silica resins, the use of affinity
resins, ion exchange chromatography and the use of
magnetic beads. Methods are described in US Patents
5057426, 4923978, EP Patents 0512767 A1 and EP 0515484B
30   and WO 95/13368, WO 97/10331 and WO 96/18731. These
patents and patent applications disclose methods of

adsorbing nucleic acids on to a solid support and then isolating the nucleic acids. The previously used methods use some type of solvent to isolate the nucleic acids and these solvents are often flammable, combustible or toxic.

EP0707077A2 describes a synthetic water soluble polymer to precipitate nucleic acids at acid pH and release at alkaline pH. The re-dissolving of the nucleic acids is performed at extremes of pH, temperature and/or high salt concentrations where the nucleic acids, especially RNA, can become denatured, degraded or require further purification or adjustments before storage and analysis.

WO 96/09116 discloses mixed mode resins for recovering a target compound, especially a protein, from aqueous solution at high or low ionic strength, using changes in pH. The resins have a hydrophobic character at the pH of binding of the target compound and a hydrophilic and/or electrostatic character at the pH of desorption of the target compound.

Blood is one of the most abundant sample sources for DNA analysis as blood samples are routinely taken for a wide range of reasons. However because of the viscous and proteinaceous nature of blood using known DNA extraction methods it has proved difficult to accomplish using automation due to the difficulties of handling large volumes containing relatively small amounts of DNA. Hitherto nucleic acid extraction has been partially automated only by using hazardous reagents and slow processing times.

I have now devised an improved method for the extraction
of nucleic acids and other biomolecules from blood and
other biological materials, and other samples containing
5   nucleic acid

According to the invention there is provided a method for
the extraction of biomolecules from biological material
which method comprises contacting the biological material
10   with a solid phase which is able to bind the biomolecules
to it at a first pH and then extracting the biomolecules
bound to the solid phase by elution using an elution
solvent at a second pH.

15   In particular there is provided a method for extracting
nucleic acid from a sample containing nucleic acid, which
method comprises: contacting the sample with said solid
phase at a first pH at which the solid phase has a
positive charge and will bind negatively charged nucleic
20   acid; and then releasing the nucleic acid at a higher pH
at which the solid phase possesses a neutral, negative or
less positive charge than at the first pH.

Generally the solid phase will possess an overall
25   positive charge, that is the sum of all positive and
negative charges on the solid phase as a whole is
positive. It is possible (though not preferred),
however, that the solid phase as a whole could be
negatively charged, but have areas of predominantly
30   positive charge to which the nucleic acid can bind. Such
solid phases are within the scope of the invention.

The change in the charge of the solid phase is referred to herein as "charge switching" and is accomplished by the use of a "charge switch material" in, on or as the
5   solid phase.

The charge switch material comprises an ionisable group, which changes charge to according to the ambient conditions. The charge switch material is chosen so that
10 the pKa of the ionisable group is appropriate to the conditions at which it is desired to bind nucleic acid to and release nucleic acid from the solid phase. Generally, nucleic acid will be bound to the charge switch material at a pH below or roughly equal to the
15 pKa, when the charge switch material is positively charged, and will be released at a higher pH (usually above the pKa), when the charge switch material is less positively charged, neutral, or negatively charged.

20 The present invention is more particularly directed to the use of charge switch materials which allow binding and/or releasing (especially releasing) of the nucleic acid to occur under mild conditions of temperature and/or pH and/or ionic strength.
25
Generally the charge switch material will change charge because of a change in charge on a positively ionisable group from positive to less positive or neutral, as the pH is increased in a range spanning or close to the pKa
30 of the positively ionisable group. This may also be combined with a change of charge on a negatively

ionisable group from neutral or less negative to more negative. In an alternative embodiment (described below), however, the charge switch material comprises a material which is positively charged at both pH values

5 (such as a metal oxide) and a negatively ionisable group, the charge of which becomes more negative as the pH is increased in a range spanning or close to its pKa.

The charge switch material may comprise an ionisable

10 group having a pKa between about 3 and 9. For positively ionisable groups, the pKa is more preferably at least about 4.5, 5.0, 5.5, 6.0 or 6.5 and/or at most about 8.5, 8.0, 7.5 or 7.0. A particularly preferred pKa for a positively ionisable group is between about 5 and 8; even

15 more preferred is a pKa between about 6.0 and 7.0, more preferably between about 6.5 and 7.0. The pKa for negatively ionisable groups is preferably between about 3 and 7, still more preferably between about 4 and 6, further preferably approximately at the pH at which it is

20 desired to bind nucleic acid.

Materials having more than one pKa value (e.g. having different ionisable groups), or combinations of materials having different pKa values, may also be suitable for use

25 as charge switch materials in accordance with the invention, provided that at a first (lower) pH the material(s) possess(es) a positive charge and that at a higher pH the charge is less positive, neutral or negative.

30

Generally a charge switch will be achieved by changing
the pH from a value below to a value above the pKa of the
or an ionisable group. However, it will be appreciated
that when the pH is the same as the pKa value of a

5   particular ionisable group, 50% of the individual
ionisable groups will be charged and 50% neutral.
Therefore, charge switch effects can also be achieved by
changing the pH in a range close to, but not spanning,
the pKa of an ionisable group. For example, at the pKa

10   of a negatively ionisable group, such as a carboxy group
(pKa typically around 4), 50% of such groups will be in
the ionised form (e.g. COO⁻) and 50% in the neutral form
(e.g. COOH). As the pH increases, an increasing
proportion of the groups will be in the negative form.

15

Preferably the binding step is carried out at a pH of
below the pKa of the ionisable group, or (though this is
not preferred) within about 1 pH unit above the pKa.
Generally the releasing step is carried out at a pH above

20   the pKa of the ionisable group, preferably at a pH
between 1 and 3 pH units above the pKa.

Prior art methods, such as those disclosed in EP0707077,
often use high pH to release the nucleic acid, for

25   example using strong bases such as NaOH. Such high pH
can cause depurination of nucleic acid, leading to the
problems of imperfect replication, which can impede
subsequent use of the nucleic acid, e.g. in detection
and/or amplification techniques such as Southern or

30   northern blotting or PCR.

The use of strong bases, or weak bases in combination
with heating, again as in EP0707077, can also lead to
degradation of RNA (especially at pH values of 10 or
above), and denaturation of double stranded DNA (i.e.
5   irreversible conversion of DNA from the double stranded
form at least partially into the single stranded form),
which can lead to a lack of specific binding in PCR.

The appropriate choice of pKa value(s) in accordance with
10   the invention allows the step of releasing DNA from the
solid phase to be performed under mild conditions, unlike
in the prior art. As used herein, the term "mild
conditions" generally means conditions under which
nucleic acid is not denatured and/or not degraded and/or
15   not depurinated, and/or conditions which are
substantially physiological.

Preferably the releasing step is performed at a pH of no
greater than about pH 10.5, more preferably no greater
20   than about pH 10.0, 9.8, 9.6, 9.4, 9.2, 9.0, 8.9, 8.8,
8.7, 8.6 or 8.5. Depending on the pKa(s) of the charge
switch material, the releasing step may even be performed
at lower pH values, such as 8.0, 7.5 or 7.0. Preferably
the releasing step is carried out in the substantial
25   absence of NaOH, preferably also the substantial absence
of other alkali metal hydroxides, more preferably the
substantial absence of strong mineral bases. Substantial
absence may mean that the concentration is less than
25mM, preferably less than 20mM, more preferably less
30   than 15mM or 10mM.

The desired change in pH can be achieved by altering the
ionic strength of the solution and/or the temperature,
since pH is dependent on both these factors. However,
neither high temperature nor high ionic strength are

5   generally compatible with the desired mild conditions,
and accordingly, the change in pH is preferably not
achieved by large changes in ionic strength or
temperature. Moreover, increasing ionic strength
increases competition of charged species with the nucleic

10 acid for binding to the solid phase, so can assist in
releasing the nucleic acid. Small changes of ionic
strength are therefore acceptable and may be used in
conjunction with the change in pH to release the nucleic
acid, preferably within the limits and ranges given

15 below.

Preferably the temperature at which the releasing step
performed is no greater than about 70°C, more preferably
no greater than about 65°C, 60°C, 55°C, 50°C, 45°C or 40°C.

20 More preferably, such temperatures apply to the entire
process. The releasing step, or the entire process, may
even be performed at lower temperatures, such as 35°C,
30°C or 25°C.

25 Furthermore, the releasing step preferably occurs under
conditions of low ionic strength, suitably less than 1M
or 500 mM, preferably less than 400mM, 300mM, 200mM,
100mM, 75mM, 50mM, 40mM, 30mM, 25mM, 20mM or 15mM. It
may even be below 10mM. The ionic strength may be at

30 least about 5mM, more preferably at least about 10mM.

More preferably, these ionic strengths also apply to the binding step.

PCR is sensitive to pH and the presence of charged
5   contaminants. In particularly preferred embodiments, the releasing step is performed using reagents suitable for storing nucleic acid (such as a commercially available storage buffer, e.g. 10mM Tris.HCl, pH8.0-8.5, optionally in the presence of 1mM EDTA), or using reagents suitable
10   for use in a procedure to which the nucleic acid is to be subjected (such as a PCR buffer, e.g. 10mM Tris.HCl, 50mM KCl, pH 8.5).

Common previously known nucleic acid extraction processes
15   require a step of diluting the elution product containing nucleic acid, to make the solution suitable for e.g. PCR. Preferably the present invention substantially avoids diluting the released nucleic acid.

20   Preferably the step of binding DNA occurs under mild conditions, suitably at a pH of no less than 3.0, preferably no less than 3.5, 4.0, 4.5 or 5.0. Previous methods have used high concentrations of chaotropic agents, such as 8M guanidine. Such conditions may not be
25   necessary in the practice of the present invention, in which the binding step preferably occurs in solution having a total concentration of 1M or less. More preferred temperatures and ionic strengths are as detailed above for the releasing step.

30

The use of such mild conditions for the release of
nucleic acid is especially useful for extracting small
quantities of nucleic acid, as the extracted DNA or RNA
can be added directly to a reaction or storage tube
5   without further purification steps (e.g. steps
necessitated by the use of high ion concentrations in
prior art methods), and without the need to dilute high
ionic strength (as is the case with prior art methods
using high ionic strength to elute the nucleic acid).
10   Therefore loss of nucleic acid through changing the
container, imperfect recovery during purification steps,
degradation, or denaturation, and dilution of small
amounts of nucleic acid can be avoided. This is
particularly advantageous when a nucleic acid of interest
15   is present in a sample (or is expected to be present) at
a low copy number, such as in certain detection and/or
amplification methods.

Broadly speaking, preferred chemical species for use as
20   charge switch materials in accordance with the invention
comprise a positively ionisable nitrogen atom, and at
least one, but preferably more than one, electronegative
group (such as a hydroxy, carboxy, carbonyl, phosphate or
sulphonic acid group) or double bond (e.g. C=C double
25   bond), which is sufficiently close to the nitrogen atom
to lower its pKa. It has been found that such molecules
tend to have suitable pKa values for the extraction of
nucleic acid under mild conditions according to the
present invention. Preferably at least one (but more
30   preferably more than one) electronegative group is
separated from the ionisable nitrogen by no more than two

atoms (usually carbon atoms). Hydroxyl groups are
particularly preferred electronegative groups
(particularly when several hydroxyl groups are present,
e.g. in polyhydroxyl amines, such as Tris $(C(CH_2OH)_3-NH_2)$
5   or Bis-Tris (see below)), as they (1) lower the pKa of
the nitrogen atom (e.g. amine group, e.g. from about 10
or 11) to a suitable value around neutral (i.e. pKa of
about 7), (2) allow the species to remain
soluble/hydrophilic above the pKa, when the nitrogen atom
10   of the amine group loses its positive charge, (3) provide
a site for covalent linkage to a solid substrate, e.g. a
polycarboxylated polymer (such as polyacrylic acid), and
(4) are uncharged at pH values suitable for the releasing
step and at which procedures such as PCR are performed
15   (typically pH 8.5); the presence of charged species can
interfere with PCR especially. Especially preferred are
chemical species having an ionisable nitrogen atom and at
least 2, 3, 4, 5 or 6 hydroxyl groups.

20   Many standard, weakly basic, buffers are ideal chemical
species to provide the ionisable groups of charge switch
materials, as they have pKa values close to neutral (i.e.
7).

25   For use as a charge switch material, chemical species
comprising ionisable groups can be immobilised onto solid
supports (e.g. beads, particles, tubes, wells, probes,
dipsticks, pipette tips, slides, fibers, membranes,
papers, celluloses, agaroses, glass or plastics) in a
30   monomeric or polymeric form via adsorption, ionic or
covalent interactions, or by covalent attachment to a

polymer backbone which is in turn immobilised onto the
solid support. Alternatively, they can be incorporated
into solid, insoluble forms (with or without attachment
to a polymer backbone) which inherently exhibit charge
5   switching, e.g. beads, particles, tubes, wells, probes,
dipsticks, pipette tips, slides, fibers, membranes or
plastics.

Solid phase materials, especially beads and particles,
10   may be magnetisable, magnetic or paramagnetic. This can
aid removal of the solid phase from a solution containing
the released nucleic acid, prior to further processing or
storage of the nucleic acid.

15   Preferably the weakly basic buffers are biological
buffers, i.e. buffers from the class of buffers commonly
used in biological buffer solutions. Examples of
biological buffers may be found in commercial chemical
catalogues, such as the Sigma catalogue.
20

Leaching (i.e. transfer from the solid phase into
solution in the liquid phase) of chemical species used to
provide ionisable groups in ion exchange resins is a
virtually inevitable phenomenon to some extent,
25   especially when the species are attached to the solid
phase by adsorption. Such leaching typically causes
impurity in the resultant product, which can lead to
significant problems, particularly if the resultant
product is intended to be used in PCR (and especially
30   when the species are charged). The use of biological
buffers to provide the ionisable groups in charge switch

materials can avoid this problem, since leaching of such
buffers into the liquid phase will generally not
significantly affect the nucleic acid, nor any downstream
processes such as PCR to which it might be subjected.

5   Indeed, many biological buffers are routinely used in PCR
buffers, storage buffers and other buffer solutions.

In a particularly preferred embodiment, the releasing
step takes place in a buffer solution containing the same

10   biological buffer that is used in, as or on the charge
switch material.

Examples of suitable biological buffers for use in charge
switch materials in accordance with the invention, and

15   their pKa values, are as follows:

N-2-acetamido-2-aminoethanesulfonic acid ‡‡ (ACES),
pKa 6.8;

N-2-acetamido-2-iminodiacetic acid ‡‡ (ADA), pKa
6.6;

20   amino methyl propanediol † (AMP), pKa 8.8;

3-1,1-dimethyl-2-hydroxyethylamino-2-hydroxy
propanesulfonic acid † (AMPSO), pKa 9.0;

N,N-bis2-hydroxyethyl-2-aminoethanesulfonic acid ††
(BES), pKa 7.1;

25   N,N-bis-2-hydroxyethylglycine † (BICINE), pKa 8.3;

bis-2-hydroxyethyliminotrishydroxymethylmethane ‡‡
(Bis-Tris), pKa 6.5;

1,3-bistrishydroxymethylmethylaminopropane ‡‡ (BIS-
TRIS Propane), pKa 6.8;

30   4-cyclohexylamino-1-butane sulfonic acid (CABS), pKa
10.7;

3-cyclohexylamino-1-propane sulfonic acid (CAPS), pKa 10.4;

3-cyclohexylamino-2-hydroxy-1-propane sulfonic acid (CAPSO), pKa 9.6;

2-N-cyclohexylaminoethanesulfonic acid (CHES) pKa 9.6;

3-N,N-bis-2-hydroxyethylamino-2-hydroxypropanesulfonic acid †† (DIPSO), pKa 7.6;

N-2-hydroxyethylpiperazine-N-3-propanesulfonic acid †† (EPPS or HEPPS), pKa 8.0;

N-2-hydroxyethylpiperazine-N-4-butanesulfonic acid † (HEPBS), pKa 8.3;

N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid †† (HEPES), pKa 7.5;

N-2-hydroxyethylpiperazine-N-2-propanesulfonic acid †† (HEPPSO), pKa 7.8;

2-N-morpholinoethanesulfonic acid ‡ (MES), pKa 6.1;

4-N-morpholinobutanesulfonic acid †† (MOBS), pKa 7.6;

3-N-morpholinopropanesulfonic acid †† (MOPS), pKa 7.2;

3-N-morpholino-2-hydroxypropanesulfonic acid ‡‡ (MOPSO), pKa 6.9;

piperazine-N-N-bis-2-ethanesulfonic acid ‡‡ (PIPES), pKa 6.8;

piperazine-N-N-bis-2-hydroxypropanesulfonic acid †† (POPSO), pKa 7.8;

N-trishydroxymethyl-methyl-4-aminobutanesulfonic acid † (TABS), pKA 8.9;

N-trishydroxymethyl-methyl-3-aminopropanesulfonic acid †† (TAPS), pKa 8.4;

3-N-trishydroxymethyl-methylamino-2-
hydroxypropanesulfonic acid †† (TAPSO), pKa 7.4;

N-trishydroxymethyl-methyl-2-aminoethanesulfonic
acid †† (TES), pKa 7.4;

5   N-trishydroxymethylmethylglycine † (TRICINE), pKa
8.1; and

trishydroxymethylaminomethane † (TRIS), pKa 8.1;

histidine*, pKa 6.0, and polyhistidine ‡‡;

imidazole*, pKa 6.9, and derivatives* thereof (i.e.
10   imidazoles), especially derivatives containing hydroxyl
groups**;

triethanolamine dimers**, oligomers** and
polymers**; and

di/tri/oligo amino acids**, for example Gly-Gly, pKa
15   8.2; and Ser-Ser, Gly-Gly-Gly, and Ser-Gly, the latter
three having pKa values in the range 7-9.

In a preferred embodiment, the buffers marked above with
an asterisk (*) are not considered to be biological
20   buffers for the purposes of the invention (whether or not
they are designated as such in any chemical catalogue).
In a more preferred embodiment, those marked with two
asterisks (**) are also not considered to be biological
buffers. Preferred biological buffers are marked with a
25   dagger (†), more preferred buffers are marked with two
daggers (††), still more preferred buffers are marked
with a double dagger (‡) and most preferred buffers are
marked with two double daggers (‡‡).

30   These and other chemical species comprising ionisable
groups may be coated as monomers onto a solid phase

support using covalent, ionic or adsorption interactions. Additionally or alternatively, they may be coated onto such solid phase supports in polymeric form (preferably following condensation polymerisation), for example by

5 adsorption onto a negatively charged surface (e.g. a surface having exposed COOH or SO_3 groups), or by covalent attachment. Additionally or alternatively, the chemical species containing ionisable groups may be attached to a polymer (see below) which is then attached

10 to a solid support, e.g. by adsorption or covalent attachment.

Preferably the chemical species or polymer backbones are covalently coupled to the solid support via a hydroxyl

15 group or other group so that the ionisable group having the desired pKa value (usually, but not limited to, a nitrogen atom) remains capable of binding and releasing nucleic acid.

20 Biological buffers and other chemical species comprising positively ionisable groups may be used in conjunction with a chemical species containing a negatively ionisable group which has a suitable pKa, preferably in the ranges described above. For example a biological buffer (having

25 one or more positively ionisable nitrogen atoms) may be attached to a polymer or other solid phase material which has exposed carboxy groups even after attachment of the biological buffer. Such a material may bind nucleic acids at a low pH when few of the carboxy groups are

30 negatively charged (i.e. few are in the COO^- form, most being in the COOH form) and most of the ionisable

nitrogen atoms are positively charged. At higher pH the negative charge is stronger (i.e. a greater proportion of carboxy groups are in the COO^- form) and/or the positive charge is weaker, and the nucleic acid is repelled from
5   the solid phase.

Chemical species containing ionisable groups (such as the biological buffers listed above) can be attached to a polymer backbone using known chemistries. For example a
10 chemical species containing a hydroxyl group can be attached using carbodiimide chemistry to a carboxylated polymer backbones. Other chemistries include can be employed by someone skilled in the art using other polymer backbones (e.g. based on polyethylene glycol
15 (PEG) or carbohydrate) using a range of standard coupling chemistries (see e.g. Immobilised Affinity Ligand Techniques, Greg T. Hermanson, A. Krishna Mallia and Paul K. Smith, Academic Press, Inc., San Diego, CA, 1992, ISBN 0123423309, which is incorporated herein by
20 reference in its entirety.)

Alternatively, the chemical species containing ionisable groups can be polymerised without a backbone polymer, using cross-linking agents, for example reagents that
25 couple via a hydroxy group (e.g. carbonyldiimidazole, butanediol diglycidyl ether, dialdehydes, diisothiocyanates). Polymers may also be formed by simple condensation chemistries to generate polymeric amino acids with the appropriate pKa e.g. Gly-Gly.
30

Preferably such immobilisation, attachment and/or
polymerisation of the chemical species containing the
ionisable group does not affect the pKa of the ionisable
group, or leaves it in the desired ranges given above.

5   For example it is generally preferred not to couple or
polymerise the chemical species via a positively
ionisable nitrogen atom (in constrast for example to
WO97/2982). In the practice of the invention, it is
especially preferred to immobilise, attach and/or

10   polymerise the chemical species via an hydroxyl group.

A preferred polymeric material is a dimer or oligomer of
Bis-Tris, or a material formed by attaching a plurality
of Bis-Tris molecules to a polyacrylic acid backbone,

15   e.g. by reacting Bis-Tris monomer with polyacrylic acid
using 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC).
The polymer can then be easily separated from the
reactants using dialysis against a suitable reagent or
water. Preferably the polyacrylic acid has molecular

20   weight of between about 500 and 5 million or more. More
preferably it has a molecular weight of between 100,000
and 500,000.

The nature of the resultant Bis-Tris/polyacrylic acid

25   molecule will depend on the ratio of the coupled
components, since the polymer will have different
properties depending on the proportion of the acrylic
acid groups that are modified with Bis-Tris, for example
it is desirable for some carboxy groups to remain

30   unmodified, as the presence of these will not prevent the
Bis-Tris from binding nucleic acid at low pH (especially

if the Bis-Tris is in excess), but their negative charge
at higher pHs will assist with release of the nucleic
acid. For use in the present invention, the molar ratio
of Bis-Tris:carboxy groups (before attachment) is
5   preferably between 5:1 and 1:5, more preferably between
3:1 and 1:3, still more preferably between 2:1 and 1:2,
further preferably between 1.5:1 and 1:1.5, and most
preferably about 1:1.

10   The presence of high residual charge (i.e. charged
species present in solution along with the extracted
nucleic acid) may adversely affect the analysis of
nucleic acids by PCR, or interfere with the binding of
primers, dNTPs or polymerase to the nucleic acid, or to
15   the sequestration of Mg^{2+} ions, which are essential to
PCR. It is particularly preferable to avoid residual
positive charge.

Preferred materials for use in the invention, such as the
20   biological buffers described above, possess minimal
residual positive charge (preferably minimal residual
charge) at the pH at which the nucleic acid is released,
and/or at pHs 8-8.5, making interference with or
inhibition of downstream processes unlikely.
25
Patent application PCT/GB00/02211, of the same inventor,
discloses certain methods within the scope of the present
invention and is incorporated herein by reference in its
entirety as exemplification of the present invention (in
30   all its aspects - see below for other aspects of the
invention). In particular, it discloses a method for the

extraction of biomolecules from biological material which
method comprises contacting the biological material with
a solid phase which incorporates histidine or a
polyhistidine which will tend to bind nucleic acids at
5   low pH and then extracting the biomolecules bound to the
solid phase by elution using an elution solvent which
will then release the bound nucleic acids when the pH is
increased.

10 An alternative embodiment of the present invention uses a
material which is positively charged across a wide pH
range, such as 0-12 or 0-14 (e.g. an electropositive
substance such as a metal oxide, metal, strong or weak
base, which lacks a pKa value, or for which the pKa value
15 is at an extreme of high pH. Such a positively charged
material is combined with negatively ionisable material
having a pKa intermediate between the pH values at which
it is desired to bind and release nucleic acid, or
slightly below the pH at which it is desired to bind
20 nucleic acid. This combination of materials allows
nucleic acid to be bound at certain pH values, around and
below the pKa of the negatively ionisable material, when
there are fewer negatively charged groups, but allows the
nucleic acid to be released when the pH is increased and
25 a greater number of the ionisable groups are negatively
charged. For example, the combination of iron II,III
oxide and polycarboxylates (see Examples) binds nucleic
acid at pH 4, when a relative scarcity of negative
charges allowing the positively charged iron oxides to
30 bind the nucleic acid. When the pH is increased to
around 8, a large proportion of the carboxy groups become

negatively charged and, despite the remaining presence of positive charges on the iron oxides, the reduction in overall positive charge allows the nucleic acid to be released.

5

Further examples of charge switching molecules for nucleic acid purification are based on detergents or surfactants that have a hydrophobic portion and a hydrophilic portion which comprises a positively
10 ionisable group with a suitable pKa, e.g. decyl methyl imidazole or dodecyl-Bis-Tris. These detergents/surfactants can be adsorbed onto surfaces e.g. plastic via their hydrophobic portions and the hydrophilic (ionisable) portions can be used to capture
15 nucleic acid.

Another family of suitable materials for capture and easy release of nucleic acids are carbohydrates e.g. glucosamine, polyglucosamine (including chitosans),
20 kanamycins and their derivatives i.e. sugar ring based structures containing one or more nitrogen atoms surrounded by hydroxyl groups which may also contain other groups such as acetate or sulphate groups to provide a suitable pKa for binding and release of nucleic
25 acids.

Another group of materials with suitable pKa values are nucleic acid bases, e.g. cytidine (pKa 4.2). These can be immobilised via hydroxy groups to a polymer or solid
30 phase carboxy group using carbodiimides.

A still further group of materials having members with suitable pKa values are heterocyclic nitrogen-containing compounds. Such compounds may be aromatic or aliphatic and may be monomers, oligomers or polymers, such as

5   morpholine-, pyrrole-, pyrrolidine-, pyridine-, pyridinol-, pyridone-, pyrroline-, pyrazole-, pyridazine-, pyrazine-, piperidone-, piperidine-, or piperazine-containing compounds, e.g. polyvinylpyridine. Such compounds may be substituted with electronegative

10   groups to bring the pKa value(s) of the ionisable nitrogen atom(s) into an acceptable range, e.g. as defined above. However, in some compounds this may not be necessary, the pKa already being in such a range.

15   Preferred materials for use in accordance with the invention are hydrophilic, for example comprising charge switch materials which are (or which comprise chemical species which before immobilisation or polymerisation are) water soluble.

20

Once a suitable solid phase has been prepared, comprising a charge switch material, repeated capture and release of nucleic acids can be performed by adjusting the pH up or down. Thus sequential reactions or analysis can be

25   performed on the nucleic acids using the same solid phase. For example, DNA can be isolated from a biological sample using a PCR tube comprising a charge switch material. Then, following PCR, the amplified DNA product may be isolated from the buffer constituents or

30   primers by adjusting the pH in the same tube.

Particularly preferred solid phase materials are non-porous. Porous supports are commonly used for isolating proteins, which can be trapped in the pores of the support. However, nucleic acids tend to be too big to enter into pores of commonly used such supports, and will therefore become bound to the surface of the support, potentially trapping impurities in the pores.

The method can be used to separate single stranded RNA or DNA from double stranded DNA, because of the different charge densities on single and double stranded molecules, by appropriate manipulation of the pH or salt concentration. Typically, single stranded molecules will be released from binding to the solid phase at a lower pH than double stranded molecules.

In some circumstances, for example for the construction of gene chips, and for the preparation of probes, it may be desirable to produce single stranded DNA. Manipulation of pH and/or ionic strength can assist in purification and release of single stranded nucleic acid. The method of the invention may comprise a prior step of converting double stranded nucleic acid in the sample to single stranded nucleic acid (preferably using a strong base, e.g. 100mM NaOH, or a weak base at high temperature, e.g. 60-100°C). The solid phase material is preferably then added simultaneously with a buffer which changes the pH of the sample to the pH for binding single stranded nucleic acid (typically a pH of 4-7).

The materials described herein may also be employed to
capture nucleic acids in the liquid phase where binding
leads to a cross-linked lattice large enough to separated
from the liquid phase, e.g. by filtration or
5   centrifugation.

Accordingly, in a second aspect, the present invention
provides a method for extracting nucleic acid from a
sample containing nucleic acids, which method comprises:
10   contacting the sample with a charge switch material at a
first pH at which the charge switch material has a
positive charge and will bind negatively charged nucleic
acid; and then releasing the nucleic acid at a second,
higher pH at which the charge is neutral, negative or
15   less positive than at the first pH, wherein the charge
switch material is soluble at said first pH, and wherein
the combination of the charge switch material and the
bound nucleic acid is insoluble at or above said first pH
and below said second pH.
20
Preferred features of the method are as set out above,
with the exception of the charge switch material being
formed into, immobilised on, or attached to, a solid
phase material.
25
Usually the charge switch materials will be soluble at
the second pH, and will remain in solution with the
nucleic acid upon release of the nucleic acid; the use of
a weakly basic buffer (optionally bound to a soluble
30   backbone, e.g. polyacrylic acid) as the charge switch

material can avoid problems of contamination as described above.

The methods of the invention preferably include one or more washing steps between the binding and releasing steps. Such (a) washing step(s) will generally be carried out at said first pH, or a pH above said first pH but lower then said second pH, such that the nucleic acid is substantially not released during the washing step(s).

As has been indicated previously, the methods of the invention are particularly suitable for extracting nucleic acid which is then stored or further processed (e.g. by PCR), particularly when the charge switch material is in the form of e.g. a tube or well in which such storage and/or processing can occur. For the avoidance of doubt, however, it is emphasised that the releasing step and any subsequent storage or processing need not be carried out as discrete steps, but can coincide, when said storage or processing occurs at a pH at which release of the nucleic acid occurs. For example, the method of the invention includes binding nucleic acid to a charge switch material coated on or otherwise provided by a PCR tube, washing the bound nucleic acid, and then without a separate releasing step commencing the PCR reaction using a PCR buffer which causes release of the nucleic acid.

In a further aspect, the present invention provides novel charge switch materials for use in the methods of the receding aspects. It further comprises the use of such

charge switch materials in such methods. All preferred features of the charge switch materials described in above in the context of the methods apply equally and independently to the present aspect of the invention 5 (i.e. preferred combinations of features may be different in relation to this aspect from the preferred combinations in relation to the method aspects).

In a further aspect, the present invention provides a 10 container (preferably a PCR or storage tube or well, or a pipette tip) coated with, comprising or formed from a charge switch material, preferably a charge switch material comprising a biological buffer.

15 The following description is directed particularly to the extraction of nucleic acid from blood, but applies also to the extraction of nucleic acid from any liquid sample, particularly biological samples or samples produced during laboratory techniques, such as PCR.
20

The method is particularly useful if the biological material is blood, but the method can be used for a range of applications substances such as plasmid and vector isolation and plant DNA extraction.
25

Preferably the cells in the blood are lysed to release nucleic acids and known lysing agents and methods can be used, such as contacting with ionic and non ionic detergents, hypotonic solutions of salts, proteases, 30 chaotropic agents, solvents, using pH changes or heat. A

method of lysing cells to isolate nucleic acid is
described in WO 96/00228.

When the biological material consists of blood the
5   samples can optionally be diluted with water or other
diluent in order to make it easier to manipulate and to
process.

Dilutions up to ten times can be used and in general more
10   dilution can be better and it is a feature of the present
invention that it allows low dilution of blood to be
possible.

The solid phase with which the blood is contacted, can be
15   a formed of a material which has a natural affinity for
nucleic acids or it can be formed of a material which has
its surface treated with an agent which will cause
nucleic acids to bind to it or increase its affinity for
nucleic acids. Suitable materials include controlled pore
20   glass, polysaccharide (agarose or cellulose), other types
of silica/glass, ceramic materials, porous plastic
materials such as porous plastic plugs which in a single
moulded part or as an insert in a standard tube,
polystyrene beads para magnetic beads etc. The size and
25   porosity is not critical and can vary and be selected for
particular applications.

Suitable means for treating the surface of the solid
phase or for derivatising it include treating it with a
30   substance which can introduce a charge e.g. a positive
charge on the surface or a hydrophilic or hydrophobic

surface on the solid phase e.g. hydroxyl groups, nitrate groups, autoreactive groups, dyes and other aromatic compounds.

5   In a preferred embodiment of the invention the solid phase will cause DNA to be bound to it at one pH in preference to contaminants in the blood sample and will allow the bound nucleic acid to be released when it is contacted with an eluant at a different pH. This system
10   can be used with a solid phase which incorporates histidine or a polyhistidine which will tend to bind nucleic acids at low pH e.g. less than 6 and will then release the bound nucleic acids when the pH is increased e.g. to greater than 8. Alternatively the nucleic acids
15   are bound at substantially neutral pH to an aminated surface and released at very high pH.

In another embodiment of the invention a plastic moulding can incorporate a binding agent e.g. in a well in a plate
20   etc. so that the binding agent is incorporated in the surface, the blood sample is then contacted with the surface so as to cause nucleic acids to be bound to the surface. The blood sample is then removed and the surface treated with an eluting agent to release the bound
25   nucleic acids. When the surface is part of a well in a multi- well plate, the total system can be readily adapted for rapid large scale sampling and extraction techniques.

30   Binding agents which can be used include charge switchable ion exchange resins using a positively charged

solid phase that can be reversed or made neutral by
changing the pH above its pKa. e.g. nucleotides,
polyamines, imidazole groups and other similar reagents
with a suitable pKa value.

5

Also, nucleic acids can be bound by intercalation using a
variety of intercalating compounds incorporated into the
solid phase e.g. actinomycin D, ethidium bromide etc.

10 In a further embodiment of the invention a plastic
surface can be modified to include functional groups. The
plastic can be any plastic used for containing samples
e.g. polypropylene. The functional groups can be
positively or negatively charged so as to bind the
15 nucleic acids in the correct buffer solution.

Alternatively the functional groups can be chemical
groups capable of covalent coupling to other ligands or
polymers.

20

When the plastic is used in a plastic moulding e.g. in a
well in a plate, or as a polymerase chain reaction (PCR)
tube, the surface characteristics of the plastic can be
suitably modified for use in the present invention by
25 including or adding the appropriate chemicals in the
moulding compound e.g. as in an injection moulding
compound.

When this is used in a PCR tube or in a deep well plate
30 the tubes or wells can be used to isolate and immobilise
small quantities of DNA or RNA generating a pure template

for subsequent PCR or other genetic analysis and
manipulation.

When the plastic is polypropylene e.g. it is in the form

5   of a thin walled PCR tube the polypropylene surface can
be modified by oxidising the surface with an oxidising
agent such as potassium permanganate and sulphuric acid
to create a carboxylated surface (COOH groups). This tube
can then be used to improve the isolation of DNA from

10 solutions or from crude samples e.g. blood. By adjusting
the pH, di-electric constant, solubility or ionic
strength the DNA or RNA can be immobilised on the walls
of the tube, washed free of contaminants, ready for PCR
or other analytical techniques.

15

The carboxy groups can be further modified by covalently
coupling an anionic group such as imidazole or
polyhistidine or any strong or weak ion exchanger, to
allow binding of nucleic acids by a charge interaction.

20 This tube could then be used to improve the isolation of
DNA from solutions or from crude samples e.g. of blood.
Again by adjusting the pH, di-electric constant, or ionic
strength the DNA or RNA can be immobilised on the walls
of the tube, washed free of contaminants, ready for PCR

25 or other analytical techniques.

The nucleic acids can be eluted with in a low salt buffer
so that it is ready for PCR or other analysis.

30 The solid phase can be contacted with a blood sample by
mixing with the solid phase in a mixing/ stirring device,

by passing the blood sample over the solid phase or the
solid phase can be paramagnetic and manipulated by a
magnetic field. Although the invention is particularly
suitable for the separation or isolation of nucleic acids
5   from blood it can be used with a range of biomolecules
particularly those that require removal of cell wall
debris or insoluble particles.

In a preferred embodiment of the invention the solid
10   phase is in granular form in a column and the blood
sample is drawn up through the column by means of a
pressure differential being applied through the column,
the blood sample is drawn up with air and the granular
solid material can become fluidised thus increasing the
15   mixing and contacting rates and minimising clogging.

The method of the invention is suitable for use in a
multi-well format when a series of extractions from
different samples can take place substantially
20   simultaneously and this will facilitate the automation of
the extraction process allowing rapid high throughput
extraction to take place and to allow combinational
chemistry to be performed. This will enable there to be a
high throughput in a standard well array e.g. an eight by
25   twelve array so that a large number of sample types can
be treated automatically at the same time.

The invention, in its various aspects, will now be
described in detail, by way of example only.
30

Example 1: Extraction of Nucleic Acids from Whole Blood

A charge switchable ion-exchanger was prepared by
covalently coupling polyhistidine to 100 (m glass beads
using glutaldehyde by mixing 1 gram of the aminated glass
5   beads with 0.01 %(v/v) glutaldehyde in 0.1M sodium
bicarbonate at pH8 containing 20mg polyhistidine. After
overnight incubation the beads were washed exhaustively
to remove non-covalently bound material and stored in
10 10mM MES, pH5 containing 0.1 % (v/v) Tween 20.

About 300mg of the 100(m derivitised glass beads were
added to a 1ml plastic column enclosed at both ends.

15 A blood sample was incubated with an equal volume of 10mM
MES pH5, containing 1% Tween 20, proteases (200(g/ml) and
1 mM EDTA. After digestion is complete the blood was
sucked up the column containing the glass beads and the
DNA became immobilised allowing the contaminating
20 proteins to pass through to waste.

The glass beads containing the immobilised DNA were
washed with a buffer comprising 10mM MES pH5, containing
1% Tween 20, and 1 mM EDTA and this was repeated until
25 the wash solution was colourless.

After washing, the beads were dried with air and DNA
eluted with a small quantity of 10mM Tris HCl, pH 8.5 and
collected in a sterile tube ready for analysis. Thus the
30 DNA were separated from the blood.

For different biomolecules, the buffer etc. can be suitably modified.

5   Example 2

One gram of carboxylated paramagnetic beads were washed in 50mM Imidazole buffer pH6 and then mixed with 100mg of polyhistidine in 50ml of 50mM Imidazole buffer pH 6. A
10   chemical coupling agent was added (EDC) at a final concentration of 5mg per ml and mixed overnight. The beads were washed in water, 0.5M sodium chloride, 1% Tween 20, 100mM Tris HCl pH 8 and stored in 10mM MES, 0.1% Tween 20 pH5.

15

To extract DNA from blood, 1mg of beads were mixed with blood diluted in 10% Tween 20 with 25mM MES, 1mM EDTA pH 5. The beads were separated with a magnet and washed by resuspending in 1mM MES, 0.1% Tween 20. To elute the DNA
20   the beads were resuspended in 10mM Tris HCl pH 8.5 and separated with magnet leaving the DNA in solution.

Example 3: Bis-Tris solid phase magnetic beads
25

200mg of carboxylated 1 μm magnetic particles were reacted in a one step procedure with 100mg of Bis-Tris and 100mg of the carbodiimide, EDC, in 50mM imidazole buffer pH6.0. Following an overnight incubation, the
30   magnetic particles were washed and used to isolate Plasmid DNA.

An alkaline lysis method was used to prepare a cleared
5ml bacterial lysate generating a supernatant containing
the plasmid in 0.5M potassium acetate, pH5. To the
5   supernatant, 2.5mg of magnetic particles were added and
mixed for 1 minute. After magnetic separation and washing
with water pH5, the pure plasmid DNA was eluted off in
200µl of 10mM Tris.HCl pH 8.5.

10   The magnetic beads were also used to extract DNA directly
from whole blood using a detergent based digestion
reagent containing proteinase K.

15   Example 4: Tricine on solid phase magnetic beads

50mg of carboxylated 1 µm magnetic particles were reacted
in a one step procedure with 50mg of Tricine and 100mg of
the carbodiimide, EDC, in 50mM imidazole buffer pH6.0.
20   Following an overnight incubation, the magnetic particles
were washed and used to isolate Plasmid DNA. An alkaline
lysis method was used to prepare a cleared 5ml bacterial
lysate generating a supernatant containing the plasmid in
0.5M potassium acetate, pH5. To the supernatant, 2.5mg of
25   magnetic particles were added and mixed for 1 minute.
After magnetic separation and washing with water pH5, the
pure nucleic acids were eluted off in 200µl of 10mM
Tris.HCl pH 8.5.

30
Example 5: Bis-Tris solid phase polystyrene beads

1 gram of carboxylated 60µm polystyrene particles were
reacted in a one step procedure with 500mg of Bis-Tris
and 500mg of the carbodiimide, EDC, in 50mM imidazole
5   buffer pH6.0. Following an overnight incubation, the
particles were washed and used to isolate plasmid nucleic
acids as described above.

10   Example 6: Bis-Tris polymer

Bis-Tris monomer was converted into a polymer by mixing
together 160mg of polyacrylic acid with a molecular
weight of 240,000, 1.6g of Bis-Tris and 1.6g of EDC in
15   50mM imidazole pH6.0. Following an overnight incubation,
the mixture was dialysed in water. The purified polymer
was then coated onto magnetic COOH beads or used in the
liquid phase to bind genomic DNA from blood. A 5ml blood
sample was centrifuged to obtain the nuclei and WBC
20   population and the resulting pellet digested with 1% SDS.
Following precipitation with potassium acetate the
cleared supernatant was mixed with either 25mg of
magnetic-Bis-Tris or about 250µg of poly-Bis-Tris as a
liquid. In both cases the captured DNA could be
25   separated, washed in water and then redissolved in 10mM
Tris HCl pH8.5 in a pure form.

Example 7: Insoluble Tris HCl polymer
30
In this example an insoluble polymer was made with

inherent charge switching properties by mixing 80mg of polyacrylic acid with 800mg of Tris HCl and 800mg of EDC in 50mM Imidazole pH6. The insoluble precipitate that formed generated a particulate solid phase that was used

5   to capture DNA and release it in a similar manner to that described in example 4 for genomic DNA.

Example 8: Immobilised poly Bis-Tris on Tips

10

A solution of poly Bis-Tris at 1mg/ml, prepared as in Example 2, in 0.1M sodium bicarbonate pH8 incubated at 60°C for 8 hours with twenty 200μl polyproplylene pipette tips. The tips were then rinsed and used to capture about

15   150ng of plasmid DNA from a cleared bacterial lysate by pumping up and down ten times. After a quick wash with water pH5, the DNA was eluted in 50μl of 10mM Tris pH 8.5.

20

Example 9: Immobilised poly Bis-Tris on PCR tubes

A solution of poly Bis-Tris at 1mg/ml, prepared as in Example 2, in 0.1M sodium bicarbonate pH8 incubated at

25   60°C for 8 hours in a 200ul PCR plate of 8x12 tubes. After rinsing, the tubes were used to bind genomic DNA from a sample prepared according to example 4. About 50ng of DNA was subsequently eluted off per tube using 10mM Tris HCl pH8.5.

30

Example 10: Charge Switch Detergents in liquid phase

A blood sample was prepared as described in Example 4 and to the resulting supernatant decyl imidazole was added at
5   pH 4 causing precipitation of the DNA. The DNA pellet was collected by centrifugation and redissolved in 10mM Tris pH 8.5.

10   Example 11: Charge Switch Detergents on solid phase

Decyl imidazole was adsorbed onto a 200ul plastic pipette tip by soaking in a 1% solution at pH4 in 0.1M sodium acetate. A blood sample was prepared as described in
15   Example 3 and the tips were used to bind the DNA by repeated pumping and sucking. After a wash with water, about 50ng of DNA was recovered in water at pH10.

20   Example 12: Polyglucosamines

10mg of low molecular weight Chitosan was dissolved in acidified water and then 50mM imidazole pH5.5, this was mixed with 100mg of carboxy 1 μm magnetic beads and with
25   20mg of the carbodiimide EDC in 50mM imidazole pH5.5. Following an overnight incubation, the beads were washed and resuspended in 10mM MES pH5. To bind genomic DNA, 2mg of magnetic particles were added to a supernatant prepared by methods described earlier in Example 1, after
30   magnetic separation, the DNA was eluted using 100mM Tris.HCl pH 9.5.

Example 13: Kanamycin

5 A solution of genomic DNA was prepared as described in
example 3. To this sample 2mg of Kanmycin was added at a
concentration of 10mg/ml. The resulting precipitate of
DNA was filtered, washed in water at pH5 and re-dissolved
in water at pH10.
10

Example 14: Magnetisable Iron Oxides in carboxylated
polystrene

15 A 5ml Plasmid mini-prep was prepared using standard
alkaline lysis reagents to generate a cleared lysate with
a potassium acetate composition of 0.5M pH4. To this
cleared supernatant, 2.5mg of commercially available 1µm
carboxylated polystyrene magnetisable particles were
20 added to bind the plasmid DNA. The particles were washed
with water at pH4 and then the DNA eluted using 10mM Tris
HCl at pH 8.5. Typical UV ratios at 260 and 280nm were
1.7-2.0, indicating pure nucleic acids with a single band
observed with standard gel electrophoresis.
25

Example 15: Titanium Dioxide in polystyrene microtitre
plates

30 A solution of DNA at 100µg per ml in 0.1M Potassium
Acetate pH4 was allowed to stand for 1 hour in a 300µl

flat bottomed microtitre plastic plate, the plastic plate
contained titanium oxide which was incorporated as a
powder in the plastic when the plate was formed. After
washing at pH4, the DNA was recovered with water at pH10
5   and 2ml measured at 260nm versus a plain polystyrene
plate with no titanium oxide. Approximately, 50ng of DNA
was recovered per 300µl well for the plate incorporating
the titanium oxide compared to zero for the plain
polystyrene plate.
10

Example 16: Cytidine coupled to magnetic beads

1 gram of carboxylated 1 µm magnetic particles were
15   reacted in a one step procedure with 500mg of Cytidine
and 500mg of the carbodiimide, EDC, in 50mM imidazole
buffer pH6.0. After thorough washing, the beads were
used to bind nucleic acids from a plasmid preparation as
described in example 1 and recovering the pure nucleic
20   acids in water at pH10.

Example 17: Polyvinyl Pyridine (PVP)

25   20mg of commercially available PVP beads was mixed with
the supernatant containing genomic DNA from a 5ml blood
extraction described in example 4. After allowing the DNA
to bind, the beads were washed with water at pH5 and the
DNA recovered using water at pH10. Ultra violet analyisis
30   at 260 and 280nm indicated a purity ratio of 1.65.

Example 18: Separation of RNA and DNA

A solution of tRNA and sheared genomic DNA was prepared
5   at 30μg per ml in 50mM Potassium acetate buffer pH6.5
with 1M sodium chloride.   Approximately 4mg of magnetic
polyhistidine beads were mixed with 1ml of the nucleic
acid solution for one minute until binding was complete.
The beads were then thoroughly washed with water at pH5.
10   To elute the bound material, the beads were mixed with
300μl of 10mM Tris.HCl, 10mM NaCl, pH8.5.   Gel analysis
showed that most of the tRNA remained in solution and was
not bound to the beads. The eluted material contained
mostly genomic DNA with little or no tRNA.
15

Example 19: DNA analysis

In all previous examples, extracted DNA was analysed by
one or more of the following:
20   (1): ultra violet (UV) analysis at 260nm and 280nm,
to provide a measure of nucleic acid concentration;
          (2): Gel electrophoresis using 1% agarose in TBE
buffer run at 60V for 20 minutes vs a commercial
preparation of DNA as control, with ethidium bromide
25   staining to measure molecular size and to provide an
estimate of quantity of the nucleic acid; or
          (3): PCR using primers specific for actin or other
ubiquitous genes, to test integrity of the nucleic acid.

30   The results are presented as (1): direct readings from
the instrument; and (2): and (3): gel pictures.

In all cases, the examples demonstrated effective
extraction of nucleic acid which was not significantly
damaged.

5